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Effect of Aeration Rate on Biosurfactin Production in a Miniaturized Bioreactor

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ABSTRACT: Recently, the production of biosurfactants in bioreactors and their use in various pharmaceutical, chemical and food industries have been developed. Optimum production is directly related to the physicochemical condition of culture medium (such as pH and temperature) and engineering parameters of bioreactors (such as aeration rate, volume of operation and the amount of energy input). Understanding the gas transfer in shaken bioreactors equipped with a sterile closure is advantageous to avoid oxygen limitation or carbon dioxide inhibition of a microbial culture. In this study, the effect of aeration rates (due to using different design closures) on the amount of biosurfactin production by *Bacillus subtilis* ATCC 6633 in a ventilation flask as a miniaturized bioreactor was investigated. The highest biosurfactin concentration (0.0485 g/L/h) was obtained in the optimum conditions in which the amount of filling volume and shaking frequency were 15 ml and 300 rpm, respectively. The specific aeration rate (q_{in}) and maximum oxygen transfer rate (OTR_{max}), were calculated 1.88 vvm and 0.01 mol/L/h, respectively. The results showed the significant biosurfactin productivity increase under non-oxygen limiting condition.

Key words: Bacillus subtilis,Biosurfactin, Aeration,Ventilation flask,Mass transfer

INTRODUCTION

Biological processes have been widely used for industrial wastewater treatment (Rahman and Al-Malack, 2012; Molanezhad, 2011; Lalevic *et al.,* 2011; Khurram, 2011; Chen *et al.,* 2011; Assassi *et al.,* 2011). Microbial surfactants or biosurfactants have considerable advantages because of their unique properties in comparision with chemical surfactants (inherent biodegradability, low toxicity, biocompatibility and structural diversity) (Banat *et al.,* 2000). The biosurfactants have been used extensively in various industries such as petrochemicals, paper industry, food industry, agriculture, pharmaceutical, environmental bioremediation and pulp production (Desai and Banat, 1997; Banat *et al.,* 2000; Davis *et al.,* 2001; Nitschke and Ferraz, 2004). Previously, the main obstacles for commercial production of bioemulsan were costly and time-consuming involved processes. To overcome the preceding problems, different solutions have been investigated such as increasing production yield by genetically strain modifications, growth optimization, design improvement of bioreactors as well as choosing inexpensive and renewable substrates (Hilton, 1999; Wei *et al.,* 2004).

Currently, an innovative system for bioprocess development, optimization and high throughput experimentations were applied. Miniaturized bioreactor technology meets this demand, which can also reduce the total experimental costs. It is widely used for screening valuable microorganisms and monitoring the physiological activity of living systems. These bioreactors are used in diverse fields such as microbiology, toxicology, genetic engineering and bioprocess development experiments (Henzler and Schedel, 1991; Hilton, 1999). The shake flasks are traditionally equipped with different types of gas-permeable closures made from cotton, cloth, paper, polymeric sponge or membrane to prevent

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contamination. These closures play an important role in the aeration of the shaken bioreactors (Schultz, 1964; Amoabediny and Büchs, 2007). Aerobic microorganisms will be oxygen-limited if the OTR (oxygen transfer rate) is lower than the oxygen uptake rate in shaken bioreactors. To avoid oxygen limitation, it is essential to have a good understanding of the gas transfer conditions. Therefore, it is necessary to evaluate the oxygen transfer rate through the sterile closure (OTR_{plug}) and gas–liquid oxygen transfer rate (OTR_{g-L}) in shaken bioreactors. The aeration rate is drastically affected by the amount of cotton density, geometry of shake flask closures and the amount of gas transfer into bioreactor (Schultz, 1964; Amoabediny and Büchs, 2007; Amoabediny *et al.,* 2009). In previous works, a model for gas transfer inside shaken bioreactors under unsteady state condition was introduced (Amoabediny and Büchs, 2007; Amoabediny *et al.,* 2009). Aerobic cells require oxygen during fermentation, which is provided by aeration, and subsequently the exhaust gases i.e. carbon dioxide is emitted (Eisentadt *et al.,* 1994). The aeration rate changes with the increase of biomass concentration. Several studies focus on the flask medium and various bioreactors have been employed in order to describe the impact of aeration on engineering parameters such as the amount of biosurfactant concentration (Eisentadt *et al.,* 1994; Wei and Chu, 1998; Mukherjee and Das, 2005; Chen *et al.,* 2006; Yeh *et al.,* 2006).

In this study, a unique type of shaken bioreactor with different design closures (f_1 and f_9; H_{pipe} and D_{pipe}) called a ventilation flask based on previous research (Amoabediny and Büchs, 2007) was applied for biosurfactin production. The innovative miniaturized bioreactor was able to eradicate main bioemulsan production obstacles based on the statistical analysis (for the first time). The operating parameters for cultivation of *Bacillus subtilis* ATCC 6633 was shaking frequency rate and filling volume as well. The target parameters for biosurfactin productivity characterization are biomass production, pH, carbon consumption, surface tension, emulsification activity, critical micelle dilution and hemolytic activity evaluation.

MATERIALS & METHODS

In this study, a series of sterile closures with different heights and diameters for the Erlenmeyer flask (250 ml; which is called ventilation flask) were utilized. The ventilation flasks of f_1 and f_9 equipped with cotton has been used to determine the effect of aeration rate on cell growth (Fig. 1). The specifications of two ventilation flasks with sterile closures are depicted in the Table 1. For corroboration of the unsteady state model to simulate the gas transfer in two ventilation flasks (f_1 and f_9), a strain of *Bacillus subtilis* ATCC 6633 was used as a model organism for biosurfactin production. The experiments were carried out using *Bacillus subtilis* ATCC 6633 to produce surfactin. The strain was grown on a medium E consisting of KH_2PO_4 (2.7 g/L), K_2HPO_4 (13.9 g/L), sucrose (10 g/L), NaCl (50 g/L), yeast extract (0.5 g/L) and $NaNO_3$ (1 g/L) in pH (6.95). This was autoclaved at 121°C for 20 minutes and 10 ml of the following solutions: $MgSO_4$ (25 g/L), and $(NH_4)_2SO_4$ (100 g/L), which are added after cooling and sterilizing. In addition, Wolin's trace metals solution was sterilized using Millipore membrane (PVDF 0.22 µm filter unit). It contains the following compounds: EDTA (0.5 g/L), $MnSO_4.H_2O$ (3 g/L), NaCl (1 g/L), $CaCl_2.2H_2O$ (0.1 g/L), $ZnSO_4.7H_2O$ (0.1 g/L), $FeSO_4.7H_2O$ (0.1 g/L), $CuSO_4.5H_2O$ (0.01 g/L), $AlK(SO_4)_2$ (0.01 g/L), $Na_2MoO_4.2H_2O$ (0.01 g/L), Boric acid (0.01 g/L), Na_2SeO_4 (0.005 g/L) and $NiCl_2.6H_2O$ (0.003 g/L) (Youssef *et al.,* 2004). Chemicals for growth medium were analytical grade.

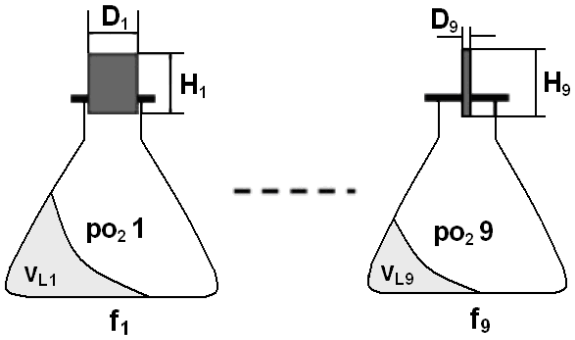

Fig. 1. The schematic drawing of the shake flasks employed in this study

Table 1. The characteristic of the sterile closures in two ventilation flasks

Flask no.	D_{pipe} *(cm)*	H_{pipe} *(cm)*
f_1	2.8	2.12
f_9	0.7	3.10

The strain was maintained on nutrient agar slant at 4°C. Preculture was produced by 1% of bacterial inoculation into the sterilized nutrient broth medium (121 °C for 15 min) and incubating in shaker incubator at 150 rpm for 24 h at 37 °C. Five % v/v of pre-culture was added to the ventlation flasks and placed in the shaker incubator (X, climo-shaker ISF1-X Kuhner) at 300 rpm at 37 °C for 3 hours. All experiments were carried out in triplicate. The culture was centrifuged at 6000 rpm for 20 minutes (Hettich Centrifuge, Universal

320, Germany) and the supernatant was separated. The biomass obtained after filtration on a 0.22 µm membrane (Whatman) was washed with distillated water and then was dried using oven at 100°C for 10 minutes. In order to determine the biomass weight, it was weighted by digital scale (Foss Tecator, Höganäs, Sweden). The pH of the supernatant was measured with a digital pH meter (METTLER TOLEDO, Seven Easy, Germany) (Carrillo and Mardaraz, 1996).

To determine the sucrose consumption, the modified phenol-sulfuric acid method was used. 2 mL of cell supernatant was mixed with 1 mL of 5% phenol solution and 5 mL of sulfuric acid, so that the vapor was formed. Then, the tubes were placed for 10 minutes in the immobilized state and were cooled in water. The absorbance intensity of the samples containing supernatant was measured by spectrophotometer apparatus (Sigma Polemic; λ= 480 nm) (Dubois *et al.*, 1956). To precipitate the biosurfactant, the pH of the filtrate was adjusted by addition of 3 M HCI (pH=2). A precipitate was collected and dried. Further purification was carried out by dissolving in deionized water. The crude surfactin was extracted three times with dichloromethane. It was dissolved in deionized water and then was centrifuged. Finally, the biosurfactant was filtered through Whatman filter paper and then was weighted (Kameda *et al.*, 1974). Surface tension (ST) and critical micelle dilutions (CMD) were measured using Du Nouy Ring Method (Cooper and Goldenberg, 1987) by digital tensiometer (K 10 ST, KRUSS, Japan) that showed the maximum force required for lifting a small ring out of supernatant. Pure water was used as standard solution. The final ST and CMD^{-1} value of each culture was the average of three readings from the same liquid. Haemolytic activity of bacterial strain was determined by inoculating blood agar (contained 5% of sheep blood), incubating plates for 24–48 h at 30 æ%C and visually inspecting for clearing zones around the colonies after this time (Jain *et al.*, 1991). All experiments were repeated in triplicate.

Emulsification activity was evaluated according to the methods described by Francy *et al.*, (1991), whereby (with a modification) 0.5, 1, 1.5, 2 ml of crude oil or other suitable hydrocarbon were added to 2 ml of the culture broth in the test tubes and mixed at high speed for 1 min using a vortex-GENIE (Francy *et al.*, 1991). The emulsion stability was determined after 24 h and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the mixture's total and multiplying by 100. All experiments were repeated in triplicate. The Minitab 5 software was used to predict the effect of parameters on response factors such as biomass production, EA, ST, CMD, and diameter of clear zone. The sterile closures of shaken bioreactors play an important role in the aeration processes (Amoabediny and Büchs, 2007). Aeration in shaken flasks is achieved with a simple gas liquid contact, supported by shaking in the rotary shaking machines such as incubator shaker (Amoabediny and Büchs, 2007). The resistance values of sterile closure can be a critical factor for oxygen supply in aerobic fermentation under certain operating conditions (Mrotzek *et al.*, 2001). Use of cotton on sterile closure in the conventional shake flasks leads to a weak understanding of the physical parameters such as gas transfer and aeration rate. Based on the new strategy developed by Amooabediny and Büchs, a kind of ventilation flask was employed in which the new relationship between gas transfer coefficient (k_{plug}) and oxygen transfer rate through the sterile closure (OTR_{plug}) was obtained. Gas transfer in this type of flasks can be affected by the coefficient of the gas liquid interface ($k_L a$) and that of sterile closure (k_{plug}) (Amoabediny and Büchs, 2007; Amoabediny *et al.*, 2009). In the aerobic process in shaken bioreactors with sterile closure, oxygen limitation occurs in the case that oxygen uptake rate (OUR_{max}) exceeds the maximum oxygen transfer rate (OTR_{max}) (Seletzky *et al.*, 2007):

$$OTR_{max} \langle OUR_{max} \quad (1)$$

It is necessary to consider the interaction of oxygen transfer rate through the sterile closure (OTR_{plug}) with oxygen transfer rate from gas to liquid phase ($OTR_{g\text{-}L}$) as well as oxygen uptake rate (OUR) which is introduced by Amooabediny and Büchs model (Amoabediny and Büchs, 2007; Amoabediny *et al.*, 2009). According to this model, partial pressure of oxygen is obtained based on the following equation:

$$\frac{\partial po_2}{\partial t} = (OTR_{plug} - OTR_{g\text{-}L}).\frac{R.T.V_L}{V_g} \quad (2)$$

Furthermore, partial pressure of oxygen in the liquid phase can be obtained as follows:

$$\frac{\partial po_{2,L}}{\partial t} = -OUR + OTR_{g\text{-}L} \quad (3)$$

Based on unsteady state gas transfer model in sulfite system commonly applied in biological system, OTR_{plug} and $OTR_{g\text{-}L}$ can be obtained from Eq. (4) and Eq. (5), respectively (Amoabediny *et al.*, 2009):

$$OTR_{plug} = k_{plug}.(\frac{1}{V_L.p_{abs}}).(po_{2,out} - po_2) \quad (4)$$

$$OTR_{g\text{-}L} = \frac{k_1 . k_L a . Lo_2 . po_2}{\left(k_1 + k_L a\right)} \quad (5)$$

Where Lo_2 is the solubility of oxygen in the solution (0.00019 mol/L/bar) which can be calculated by the method of Schumpe (Schumpe *et al.,* 1982) and k_1 is the first-order reaction constant of 2.358 1/h, reported by Hermann et al. (Hermann *et al.,* 2001).

Oxygen uptake rate by the microorganisms (*OUR*) is described by Eq. (6) where $Y_{x/o2}$, μ and X are biomass yield coefficient on oxygen consumption, specific growth rate and the value of biomass concentration, respectively. These parameters are obtained using the Model Maker software (Amoabediny and Büchs, 2007; Amoabediny *et al.,* 2009):

$$OUR = \frac{1}{Y_{X/O2}} . \mu . X \quad (6)$$

The values of OTR_{plug} and $OTR_{g\text{-}L}$ depend on the gas transfer coefficient of sterile closure (k_{plug}) and the volumetric gas–liquid mass transfer coefficient ($k_L a$) that can be obtained by Eq. (7) and Eq. (8), respectively. In Eq. (8), parameters a, b, c in accord with operating conditions can be calculated from literature data (Amoabediny *et al.,* 2009).

$$k_L a = 5.51 . 10^{-4} . V_L^{-0.85} . n^{1.15} . d_o^{0.38} \quad (7)$$

$$k_{plug} = \frac{a . OTR_{plug}}{b + OTR_{plug} + \frac{OTR_{plug}^2}{c}} \quad (8)$$

OTR_{flow} is defined as the amount of oxygen transferred by aeration into a biological or chemical system in the ventilation flask (in which the following equation is derived):

$$OTR_{flow} = \frac{1}{p_{abs} . v_{mo}} . q_{in} . \left(po_{2,out} - po_2 \right) \quad (9)$$

q_{in} is the specific aeration rate (vvm: Gas volume flow per unit of liquid volume per minute) in ventilation shake flask. According to the literature, (Amoabediny *et al.,* 2009) for an equivalent gas concentration in the headspace of both cases, gas transfer through the sterile closure and gas flow in ventilation flask, OTR_{plug} must be equal to OTR_{flow}. Hence, the following equation reveals:

$$q_{in} = \frac{v_{mo}}{v_L} . k_{plug} \quad (10)$$

The value of k_{plug} was quantified using the dependency of k_{plug} on OTR_{plug} (Eq. 8). Substituting Eq. (8) in Eq. (10); the following equation will be developed (Amoabediny *et al.,* 2009):

$$q_{in} = \frac{v_{mo}}{v_L} . \frac{a . OTR_{plug}}{b + OTR_{plug} + \frac{OTR_{plug}^2}{c}} \quad (11)$$

RESULTS & DISCUSSION

In this work, the effect of aeration rate on the amount of surfactin production by *Bacillus subtilis* ATCC 6633 in a ventilation flask as a miniaturized bioreactor was considered. This was conducted in 15 ml of filling volume and 300 rpm of shaking frequency. The experiment duration was 30 hours. It is observed that the pH value of the culture medium decreases from 6.95 to 6.11 and 6.2 in f_1 and f_9 at 14 hours, respectively (Fig. 2). Meanwhile, the biomass concentration increases during the exponential growth phase from approximately 0.85 g/L to 4.21 g/L and 3.65 g/L in f_1 and f_9, respectively. In addition, Fig. 2 depicts that biomass concentration and pH gradually starts to decrease after 14 hours.

According to Fig.3, the residual sucrose concentration in the culture in f_9 equals approximately to 0.57 g/L after 14 hours. It should be concluded that the culture does not consume all the sucrose as a whole. On the contrary, the residual sucrose concentration at f_1 reaches to approximately zero. On the other hand, the maximum biosurfactant concentration was evaluated 0.68 g/L in f_1 after 14 hours. This value equals to 0.55 g/L in f_9 at the same condition.

The effect of aeration on the surface tension is illustrated in Fig. 4. Based on Fig. 4, the surface tension decreases from 60.5 mN/m to 31.7 mN/m and 34.4 mN/m in f_1 and f_9 after 14 hours, respectively. Consequently, in f_1 the value of surface tension approximately decreases to 8.5% in comparison with f_9 at the same condition. Also, the emulsification activity increases and its maximum value reaches to 95.25% and 88.57% in f_1 and f_9 after 14 hours, respectively.

The effect of aeration on the diameter of clear zone is indicated in Fig. 5. According to Fig. 5, the diameter of clear zone in f_1 and f_9 approximately reaches 3.2 and 2.6 cm after 14 hours, respectively. On the other hand, the values of CMD^{-1} decrease from 66.80 mN/m to 35.25 mN/m and 38.17 mN/m in f_1 and f_9 after 14 hours, respectively. Consequently, the value of CMD^{-1} in f_1 decreases to 8.3% in comparison with f_9 in the same conditions. As it was expected, CMD^{-1} and diameter of clear zone in f_1 and f_9 are gradually starting to increase and decrease after 14 hours, respectively. The effect of aeartion on CMD^{-1} is obviously similar to the surface tension behaviour.

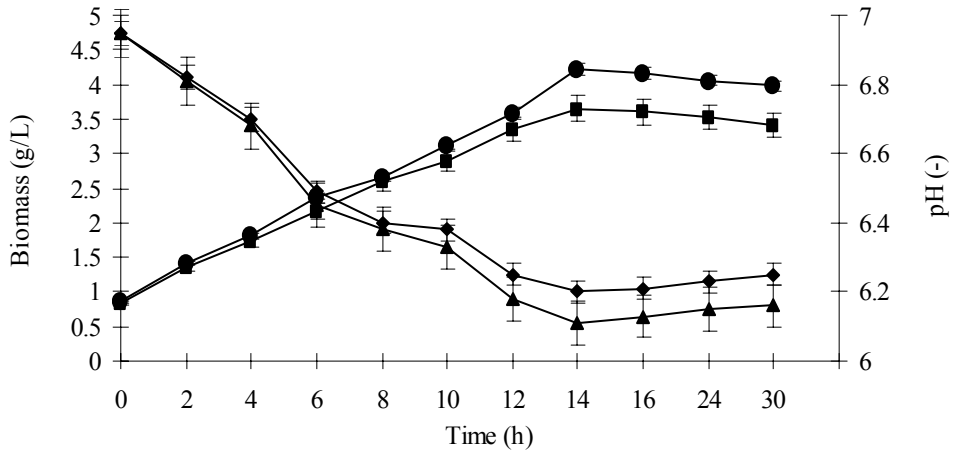

Fig. 2. Influence of aeration on the variation of biomass concentration and pH during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in 300rpm shaking frequency and 15 ml of filling volume: Biomass, f_1 (•), Biomass, f_9 (■), pH, f_1 (Δ), pH, f_9 (♦)

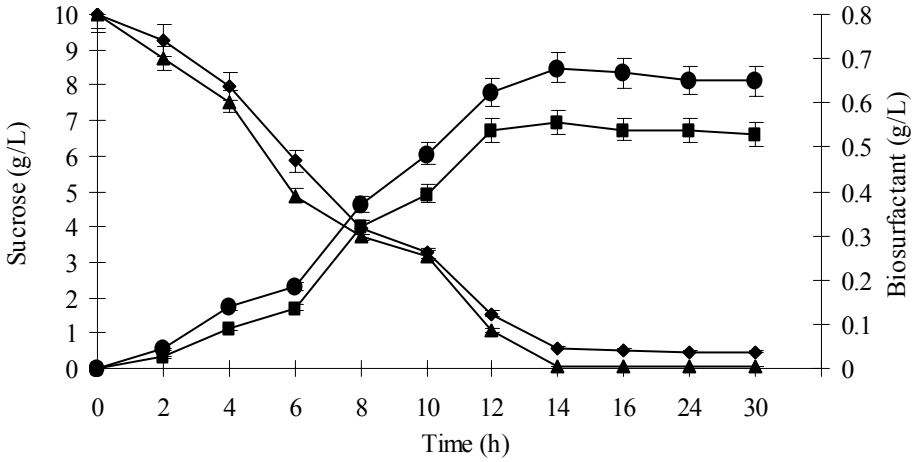

Fig. 3. Influence of aeration on the carbon source consumption and the surfactin concentration during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in 300rpm of shaking frequency and 15 ml of filling volume: Sucrose, f_1 (Δ), Sucrose, f_9 (♦), Biosurfactant, f_1 (•), Biosurfactant, f_9 (■)

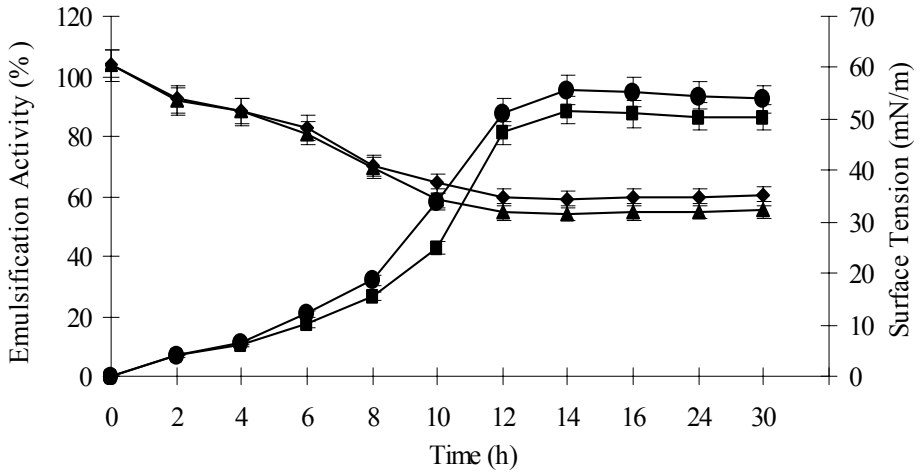

Fig. 4. Influence of aeration on the variation of emulsification activity (EA) and surface tension (ST) during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in 300rpm of shaking frequency and 15 ml of filling volume: EA, f_1 (•), EA, f_9 (■), ST, f_1 (♦), ST, f_9 (Δ)

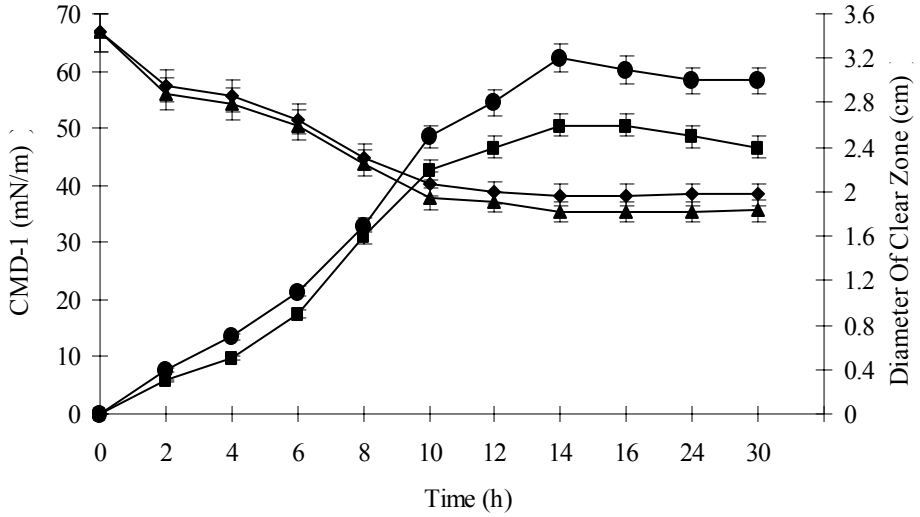

Fig. 5. Influence of aeration on the variation of CMD^{-1} and the diameter of clear zone during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in 300rpm of shaking frequency and 15 ml of filling volume: CMD^{-1}, f_1 (Δ), CMD^{-1}, f_9 (♦), Diameter, f_1 (●), Diameter, f_9 (■)

Based on the statistical analysis, whereas the f_1 shake flask was used, the effect of aeration rates on mentioned factors (pH, biomass concentration, EA, diamater size, CMD, and ST) was significant in comparison with f_2. D_{pipe} and H_{pipe} of f_1 are 2.80 cm and 2.12 cm, respectively. It shows that the effect of design parameters on biosurfaction production is important.

Based on the model and experimental data, the highest biomass concentration (0.30 g/L/h) and biosurfactant concentration (0.049 g/L/h) were obtained in f_1 ventilation flasks while the oxygen limitation occurred in f_2 shake flask. Based on the model predicted by Amoabediny and Büchs (Amoabediny and Büchs, 2007), it can be concluded that f_1 ventilation flask does not show any oxygen limitation. According to the sucrose consumption, the best yield ($Y_{p/s}$=0.068 g/g and $Y_{x/s}$=0.421 g/g) was measured in f_1, too. OTR_{max} value is 0.01 mol/L/h. In addition, by applying the mentioned model, k_La and the specific aeration rate (q_{in}) are calculated as 0.0721 1/s and 1.88 vvm, respectively while q_{in} is 0.08 vvm in f_9 ventilation flask. Table 2 indicates the values of different parameters calculated for two ventilation flasks in 300 rpm of shaking frequency and 15 ml of filling volume.

Yeh et al. (Yeh *et al.*, 2006) used a special design of a batch bioreactor in order to produce bosurfactin. k_La and q_{in} were evaluated as 0.013/s and 1.500 vvm, correspondingly in which shaking frequency and filling volume were 300 rpm and 3 liter after 60 hours, respectively. However, in our study, the time interval was 14 hours at the same shaking frequency. Also, we obtained higher values of k_La and q_{in} (0.0721/s and 1.88 vvm, respectively). In contrary to Yeh et al. results (Yeh *et al.*, 2006), in lower amount of time and working volume, maximum biosurfactan production was achieved. Biosurfactant concentration of 0.0038 g/L/h was obtained by Chen et al. (Chen *et al.*, 2006) in 24 hours. The maximum specific growth rate was 0.24 1/h whereas 0.0486 g/L/h of biosurfactin was produced within 14 hours and the maximum specific growth rate

Table 2. The values of different parameters calculated for two ventilation flasks in 300 rpm of shaking frequency and 15 ml of filling volume

Flask no.	Y_{Xmax} (g/L/h)	Y_{Pmax} (g/L/h)	μ_{max} (1/h)	O_2 consumed (mol)	OTR_{max} (mol/L/h)	k_La (1/s)	q_{in} (vvm)	Oxygen limitation
f_1	0.30	0.0486	0.2292	0.0020	0.010	0.0721	1.88	–
f_9	0.26	0.0396	0.1988	0..0017	0.008	0.0721	0.08	+

of 0.23 1/h measured. Moreover, Amoabediny et al. (Amoabediny *et al.,* 2010) used a miniaturized bioreactor (type f_1) based on previous research (Amoabediny and Büchs, 2007) for bioemulsan production enhancement. *Acinetobacter calcoaceticus PTCC* 1641 was employed in a novel synthetic medium comprised of whey and mineral salt medium (MSM) in ratio of 1:1 in a shaken flask bioreactor. The optimum inoculum size, shaking frequency rate and filling volume (V_L) using ventilation flasks were investigated that resulted in 5 vol%, 300 rpm and 15 ml, respectively. The surface tension decreased around 48% during bioemulsan production (Amoabediny *et al.,* 2010).

CONCLUSION

In shaken bioreactors equipped with a sterile closure, gas transfer understanding is advantageous to avoid oxygen limitation or carbon dioxide inhibition of a microbial culture. Based on using different design closures, the effect of aeration rates on the amount of biosurfactin production by *Bacillus subtilis* ATCC 6633 was investigated. The highest biosurfactin concentration was obtained in the optimum condition. According to the proposed model by Amoabediny and Büchs, the ongoing process improves the productivity when compared to the conventional processes. As indicated, in order to prevent oxygen limitation, the best operational condition was achieved in f_1 ventilation flask (D_{pipe}: 2.80 cm; H_{pipe}: 2.12 cm) in which the maximum biomass and biosurfactant concentration was obtained.

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REFERENCES

Amoabediny, G. H. and Büchs, J. (2007). Modeling and advanced understanding of unsteady state gas transfer in shaking bioreactors. Biotechnol. Appl. Biochem., **46**, 57–67.

Amoabediny, G. H., Ziaie, Y. and Büchs, J. (2009). Development of unsteady state for a biological system in miniaturized bioreactors. Biotechnol. Appl. Biochem., **54**, 163–170.

Amoabediny, G. H., Rezvani, M., Rashedi, H., Jokari, S., Chamanrokh, P., Mazaheri, M., Ghavami, M. and Yazdian, F. (2010). Application of a novel method for optimization of bioemulsan production in a miniaturized bioreactor. Biores. Technol., **101**, 9758–9764.

Assassi, M., Fourcade, F., Geneste, F., Floner, D., Maachi, R., Amrane, A. (2011). Combined electrochemical and biological treatment for pesticide degradation – Application to phosmet. Int. J. Environ. Res., **5 (1)**, 41-46.

Banat, I. M., Makkar, R. S. and Cameotra, S. S. (2000*). Potential* commercial *applications of microbial surfactants.* Appl. Microbiol. Biotechnol., **53**, 495–508.

Carrillo, P. G. and Mardaraz, C. (1996). Isolation and selection of biosurfactant- producing bacteria. World J. Microb. Biot., **12**, 82–84.

Chen, C. Y., Baker, S. C. and Darton, R. C. (2006). Batch production of biosurfactant with foam fractionation. Chem. Thechnol. Biotechnol., **81**, 1923–1931.

Chen, J. Huang,W., Han,J. and Cao, Sh. (2011). The Characterization and Application of Biological Remediation Technology for Organic Contaminants, Int. J. Environ. Res., **5 (2)**, 515-530.

Cooper D. G. and Goldenberg B. G. (1987). Surface-active agents from two bacillus species, Appl. Environ. Microbiol., **53**, 224–229.

Desai, J. D. and Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Rev., **61**, 47–64.

Davis, D. A., Lynch, H. C. and Varley, J. (2001). The application of foaming for the recovery of surfactin from B.subtilis ATCC 21332 cultures. Enz. Microb. Technol., **28**, 346–354.

Dubois, M., Gills, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Colorimetric method for determination of sugar and related substances. Anal. Chem., **28**, 350–356.

Eisentadt, E., Carlton, B. C. and Brown, B. J. (1994). Methods for general and molecular bacteriology. In: P. Gerhardt (Ed.), Gene Mutation, American Society Microbiology, Washington DC. 297–317.

Francy, D. S., Thomas, J. M., Raymond, R. L. and Ward, C. H. (1991). Emulsification of hydrocarbons by subsurface bacteria. J. Ind. Microbial., **8**, 237–246.

Henzler, H. J. and Schedel, M. (1991). Suitability of the shaking flask for oxygen supply to microbiological cultures. Bioproc. Eng., **7**, 123–131.

Hermann, R., Walther, N., Maier, U. and Büchs, J. (2001). Optical method for the determination of the oxygen-transfer capacity of small bioreactors based on sulfite oxidation. Biotechnol. Bioeng., **74**, 355–363.

Hilton, M. D. (1999). Small-scale liquid fermentations. In: A.L. Demain, J.E. Davies (Eds.), Manual of Industrial Microbiology and Biotechnology, American Society Microbiology, Washington DC. 49–60.

Jain, D. K., Collins-Thompson, D. L., Lee, H. and Trevors, J. T. (1991). A drop-collapsing test for screening surfactant-producing microorganisms. J. Microbial. Methods., **13**, 271–279.

Lalevic, B., Raicevic, V., Kikovic, D., Jovanovic L., Surlan, G., Jovic, J.,Talaie, A. R. and Morina, F. (2011). Biodegradation of MTBE by Bacteria Isolated From Oil

Hydrocarbons-Contaminated Environments. Int. J. Environ. Res., **5 (4)**, 827-832.

Kameda, Y., Oira, S., Matsui, K., Kanatomo, S. and Hase, T. (1974). Antitumor activity of *Bacillus natto* V. Isolation and characterization of surfactin in the culture medium of *Bacillus natto* KMD 2311. Chem. Pharm. Bull., **22**, 938–944.

Khurram, A. A. (2011). Temporal Evaluation of Biochemical Properties in Chemically Amended Wastewater Derived Biosolids. Int. J. Environ. Res., **5 (3)**, 661-672.

Molanezhad, M. (2011). The Analysis of Biotechnology Foresight Application in Iranian Organizations. Int. J. Environ. Res., **5 (4)**, 1009-1024.

Mrotzek, C. H., Anderlei, T., Henzler, H. and Büchs, J. (2001). Mass transfer resistance of sterile plugs in shaking bioreactors. Biochem. Eng. J., **7**, 107–112.

Mukherjee, A. K. and Das, K. (2005). Correlation between diverse cyclic lipopeptides production and regulation of grows and substrate utilization by Bacillus subtilis strains in a particular habitat. FEMS Microbiol Ecol., **54**, 479–489.

Nitschke, M. and Ferraz, C. (2004). Selection of microorganisms for biosurfactant production using agro industrial wastes. Braz. J. Microbiol., **35**, 81–85.

Rahman, M. M. and Al-Malack, M. H. (2012). Biochemical Kinetics of Cross flow Membrane Bioreactor Processes in the Treatment of Refinery Wastewater. Int. J. Environ. Res., **6 (1)**, 285-296.

Schumpe, A., Quicker, G. and Deckwer, W. D. (1982). Gas solubility in microbial culture media. Adv. Biochem. Eng., **24**, 1–38.

Schultz, J. S. (1964). Cotton closure as an aeration barrier in shaken flask fermentations. Appl. Microbiol., **12**, 305–310.

Seletzky, J. M., Noak, U., Fricke, J., Welk, E., Eberhard, W., Knocke, C. and Büchs, J. (2007). Scale-up from shake flasks to fermenters in batch and continuous mode with Corynebacterium glutamicum on lactic acid based on oxygen transfer and pH. Biotechnol. Bioeng., **98**, 800–811.

Wei, Y. H. and Chu, I. M. (1998). Enhancement of surfactin production in iron-enriched media by Bacillus subtilis ATCC 21332. Enz. Microb. Tech., **22**, 724–728.

Wei, Y. H., Wang, L. F. and Chang, J. S. (2004). Optimizing iron supplement strategies for enhanced surfactin production with Bacillus subtilis. Biotechnol. Prog., **20**, 979–983.

Yeh, M. S., Wei, Y. H. and Chang, J. S. (2006). Bioreactor design for enhanced carrier-assisted surfactin production with Bacillus subtilis. Proc. Biochem., **41**, 1799–1805.

Youssef, N., Duncan, K. E., Nagle, D., Savage, K. N., Knapp, R. and McInerney, M. J. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Meth. **56,** 339–347.